

# Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase

(nitrogen fixation)

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**ABSTRACT** Biological nitrogen fixation in aerobic organisms requires a mechanism for excluding oxygen from the site of nitrogenase activity. Oxygen exclusion in *Frankia* spp., members of an actinomycetal genus that forms nitrogen-fixing root-nodule symbioses in a wide range of woody Angiosperms, is accomplished within specialized structures termed vesicles, where nitrogen fixation is localized. The lipidic vesicle envelope is apparently a functional analogue of the cyanobacterial heterocyst envelope, forming an external gas-diffusion barrier around the nitrogen-fixing cells. We report here that purified vesicle envelopes consist primarily of two hopanoid lipids, rather than of glycolipids, as is the case in cyanobacteria. One envelope hopanoid, bacteriohopanetetrol phenylacetate monoester, is vesicle-specific. The *Frankia* vesicle envelope thus represents a layer specific to the locus of nitrogen fixation that is biosynthetically uniquely derived.

It is a paradox of biological nitrogen fixation in aerobic organisms that because the enzyme nitrogenase is oxygen-labile, oxygen must be excluded from the site of nitrogenase activity, whereas oxygen-dependent energy production must continue. Some mechanism to regulate oxygen diffusion to nitrogenase is therefore required for enzyme activity to occur. In actinomycetes of the genus *Frankia*, a nitrogen-fixing root-nodule endosymbiont, nitrogen fixation occurs within specialized multicellular structures termed vesicles. A vesicle is surrounded by a multilamellate, lipid-containing envelope that apparently functions as a barrier to oxygen diffusion (1-4). Several lines of evidence implicate a two-component system of oxygen exclusion in the *Frankia* vesicle, consisting of differential depletion of O<sub>2</sub> gas due to internal high rates of respiration and a vesicle-specific outer-barrier layer that reduces gas uptake (5). The existence of such a barrier layer is inferred from observed differences in kinetics of O<sub>2</sub> saturation of respiration in vesicles vs. non-vesicle cells induced to fix N<sub>2</sub> under low oxygen partial pressure (2). Moreover, external oxygen conditions regulate the deposition of envelope lamellae: although the initial signal leading to vesicle differentiation is nitrogen starvation, the thickness of the vesicle envelope varies directly with the external O<sub>2</sub> concentration (3). The effectiveness of the two-component system found in *Frankia* vesicles is illustrated by the observation (3) that *Frankia* strains fix N<sub>2</sub> at pO<sub>2</sub> levels of 70 kPa in the gas phase.

*Frankia* strains are filamentous, nitrogen-fixing actinomycetes that form root-nodule symbioses with woody plant hosts in eight Angiosperm families. Nitrogen fixation by these actinorhizal symbioses contributes a major fraction of the total nitrogen in the biosphere (5). During nitrogen starvation in culture and in several types of actinorhizal root

nodules, *Frankia* strains produce stalked, spherical vesicles that develop from hyphal branches. The vesicle envelope is deposited outside the cell wall during vesicle differentiation and covers the vesicle and the vesicle stalk (6). The envelope has been shown to contain lipid (4), which is deposited in multiple lamellae, each 3-4.5 nm in thickness (refs. 6 and 7; Fig. 1). The number of envelope lamellae and hence the thickness of the envelope as a barrier layer is regulated by external O<sub>2</sub> levels (3).

The composition of the vesicle envelope has, until now, been unknown, although it has been speculated to contain glycolipids, by analogy to the well-characterized cyanobacterial heterocyst envelope (8). To explore this assumption, fatty acid profiles of vesicles vs. vegetative cells were characterized (9), but no vesicle-specific patterns suggesting glycolipid accumulation were found. Recently we reported that hopanoid lipids compose the most abundant lipid class in nitrogen-fixing root nodule tissue with *Frankia* as microsymbiont and in *Frankia* cells in culture (10). In particular, the hopanoid bacteriohopanetetrol (C<sub>35</sub>H<sub>62</sub>O<sub>4</sub>) represents ≈30%-50% of total *Frankia* lipids. Hopanoids occur in cell membranes in a wide range of microorganisms, where these lipids contribute to membrane stability and alter phase-transition properties (11). Hopanoids are particularly important for microbial survival in extreme thermal environments (11) and have been identified as a major component of oil shales (12). In this report, we present our finding that the vesicle envelope of *Frankia* strain HFPCpI1 is composed predominantly of hopanoid lipids.

## MATERIALS AND METHODS

Purified vesicle envelopes (Fig. 1) were prepared from nitrogen-starved cultures of *Frankia* HFPCpI1 (13) by sonication and sucrose-gradient centrifugation (7). Root nodules of *Alnus rubra* cv. Bong were prepared as in ref. 10. Lipids were extracted according to methods of Bligh and Dyer (14) and separated and quantified by HPLC (10), either with a flame ionization detector or with an evaporative light-scattering detector (Varex, Burtonsville, MD; ref. 15). Total lipids were quantified gravimetrically. Individual lipids in vesicle envelopes and in root-nodule tissue were quantitated by calculating the integrated value of individual peak area as a percentage of total peak area in the HPLC-flame ionization detector chromatograms. Individual HPLC fractions were purified and analyzed with MS, as described (10).

## RESULTS

The preparations of purified vesicle envelopes from HFPCpI1 contained 84% lipid by weight. Two major lipids were present in HPLC profiles of the envelope lipids (peak 3

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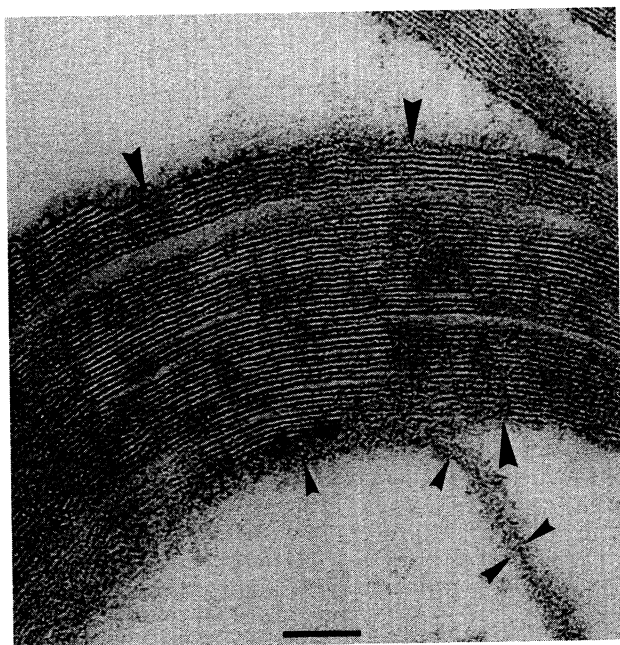


FIG. 1. Transmission electron micrograph of an isolated vesicle envelope (large arrowheads) illustrating its laminated nature. Samples were prepared for EM with potassium permanganate fixation (7) and left unstained. Approximately 48 monolayers are visible in the envelope. Portions of the bacterial cell wall (small arrowheads) remain attached to the interior of the vesicle envelope. (Bar = 50 nm.)

and peak 5 in Fig. 2a). Peak 3 consistently represented 30–50% of total envelope lipids detected, whereas peak 5 represented 30–45% of the envelope lipids. Both major lipids had retention times characteristic of intermediate-polarity lipids. Several minor overlapping peaks, with retention times intermediate between peak 3 and peak 5 (peak 4, arrows) represented  $\approx 10$ –15% of the total lipids. A minor peak with a retention time corresponding to that of free fatty acids (peak 2) represented  $\approx 5$ –8% of the vesicle envelope lipids. HPLC

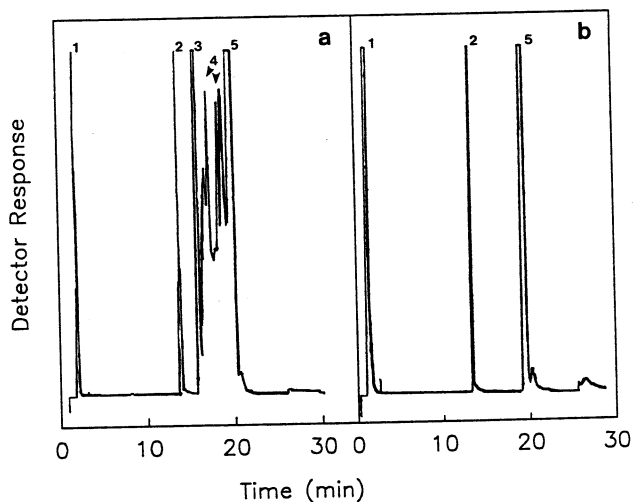


FIG. 2. HPLC chromatograms of vesicle-envelope lipids of HPFCp11 with an evaporative light-scattering detector. (a) Ten microliters of envelope lipid extract was injected. Peak 1 (1.5% of total lipids detected) represents nonpolar hydrocarbons. Retention time of peak 2 (6.8%) corresponds to that of free fatty acids. Peaks 3 and 5 are offscale: peak 3 area = 31.5%; overlapping peaks at arrowheads, 4 peaks = 16.7%; and peak 5 = 44.1% (b) Twenty microliters of envelope-lipid extract was injected after alkaline hydrolysis. Peaks 3 and 4 are not detected.

profiles of whole-vesicle preparations were similar to the profile of the purified envelope extract. Although galactolipids and phospholipids were detected previously both in nodule preparations and in vegetative *Frankia* cell extracts (10), we did not detect them in the vesicle envelope. In HPLC lipid profiles of vegetative cells, peak 5 was abundant, as reported (10), but peaks 3 and 4 were not detected. Thus peaks 3 and 4 were lipids specifically present in the vesicle envelope.

Upon alkaline hydrolysis, peaks 3 and 4 disappeared, indicating that these compounds contained probable ester linkages, whereas peak 5 remained stable (Fig. 2b). Moreover, peaks 3 and 4 appeared to be derivatives of peak 5 because no new compounds were detected after hydrolysis. Peaks 3 and 4 absorbed UV light at 205 nm, whereas peak 5 did not, suggesting that the ester groups contained at least one carbon-carbon double bond.

The mass spectrum of the per-O-acetylated compound corresponding to peak 5 was identical with that of bacteriohopanetetrol, with a molecular ion of 714, and characteristic ion fragments of  $m/z$  191, 369, and 493, as has been shown (10). The  $m/z$  of 191 and 369 are definitive for the pentacyclic moiety of hopanoid lipids. A daughter ion spectrum obtained for  $m/z$  493 from per-O-acetylated peak 5 showed successive losses of 60 (acetic acid), demonstrating that  $m/z$  493 arises from the acetylated side chain and a portion of the alicyclic ring system of the lipid.

The spectrum of per-O-acetylated peak 3 (Fig. 3) exhibited a molecular ion of  $m/z$  790, with prominent fragments of  $m/z$  91, 191, 369, and 569. The  $M_r$  is 76 mass units higher than for per-O-acetylated peak 5, suggestive of the presence of a benzene ring. Also, the peak at  $m/z$  569 appears at 76 Da greater than the peak at  $m/z$  493 in peak 5. The prominent fragment ion at  $m/z$  91 is characteristic of a phenyl-CH<sub>2</sub> group. A fast-atom-bombardment mass spectrum of peak 3 suggested a  $M_r$  of 664. A spectrum of the daughters of  $[M+H]^+$  was obtained, showing characteristic hopanoid fragments at  $m/z$  191 and 369 and  $m/z$  529  $[M+H-136]^+$ , suggesting esterification with an acid of  $M_r$  136.

The presence of  $m/z$  369 spectra from both peaks 3 and 5 demonstrated that the difference between these compounds occurs on the side chain. Moreover, the peak at  $m/z$  569 in the spectrum of per-O-acetylated peak 3 is analogous to  $m/z$  493 in per-O-acetylated peak 5 as determined by tandem MS. Daughter ions produced from fragmentation of  $m/z$  569 included  $m/z$  509 (loss of AcOH), 449 (two AcOH groups), 433 (loss of 136), 389 (three AcOH groups), and 373 (AcOH plus 136), demonstrating the presence of three acetate groups plus the ester of the  $M_r$  136 acid. The fragment at  $m/z$  433 was less abundant than the fragment corresponding to the loss of two AcOH groups ( $m/z$  449), suggesting that the  $M_r$  136 acid is esterified to the terminal OH group on the side chain and is lost as a neutral species less easily than acetic acid.

To confirm the identity of the R group, peak 3 was hydrolyzed and methylated with diazomethane; the products were then analyzed by GC/MS. At a GC retention time of 8 min, a compound eluted with molecular ion 150 and with a major ion fragment of  $m/z$  91. The spectrum was identical to the mass spectrum of the methyl ester of phenylacetic acid ( $M_r$  136 + 14).

Resonances in the <sup>1</sup>H NMR spectrum of peak 3 at  $\delta$  7.3 ppm (m) and  $\delta$  3.6 ppm (s) were consistent with the presence of a monosubstituted benzene ring and a methylene group.

Mass spectra of the HPLC fraction representing the minor overlapping peaks (Fig. 2a, peak 4, arrowheads) had prominent ion fragments of 191, 369, 493, 569, and 790 and showed that these compounds also contain bacteriohopanetetrol phenylacetate ester. However, there were ion fragments in the mass spectra, suggesting other types of carbon- and hydrogen-containing molecules, which were not identifiable. The

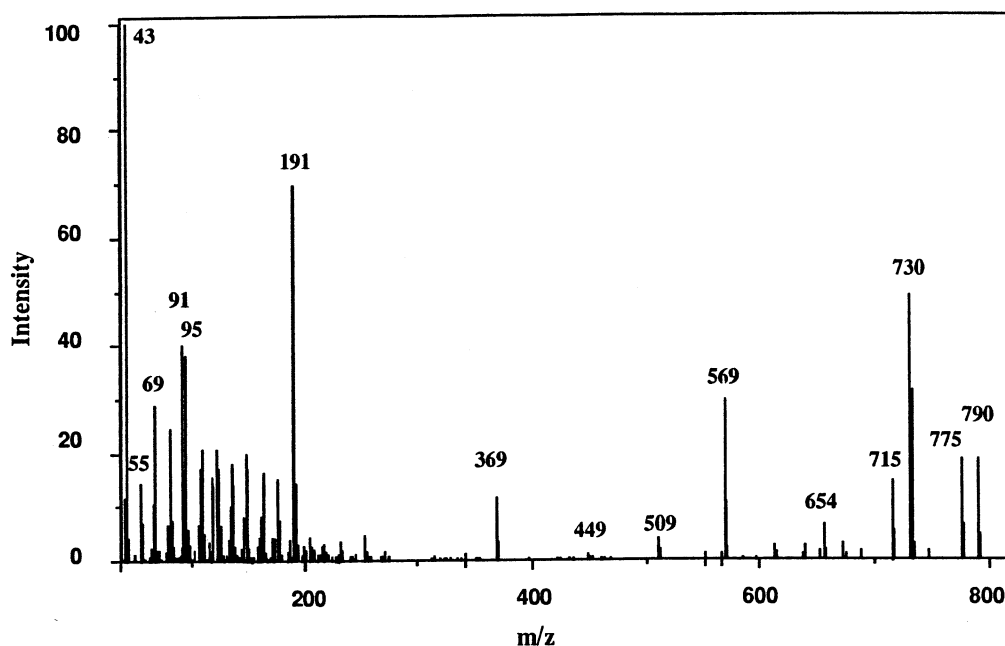


FIG. 3. Mass spectrum of purified HPLC fraction (peak 3 in Fig. 2a) after acetylation. M, 790 Da. Note major fragment ions of  $m/z$  91, 191, 369, and 569.

vesicle-envelope compounds with HPLC retention times intermediate between peaks 3 and 5 may represent secondary monoesters of the tetrol or possibly di-, tri-, or tetraesters.

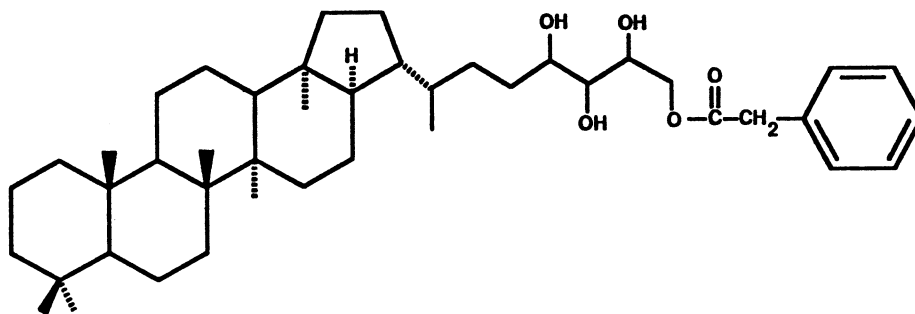
In addition to finding bacteriohopanetetrol and its phenylacetate ester in the envelope lipids of HFPCpI1, we observed HPLC peaks in lipid extracts of *Alnus rubra* nodules that had retention times similar to peaks 3–5. Mass spectra of purified nodule lipid fractions with HPLC retention times corresponding to envelope peak 3 and peak 5 were identical to spectra obtained for the corresponding envelope fractions. In these nodule preparations, bacteriohopanetetrol and its phenylacetate ester composed  $20 \pm 1.8\%$  (SE) and  $16 \pm 0.7\%$  (SE), respectively, of the total nodule lipid mass ( $n = 5$ ), representing by far the most abundant lipids in the nodule-tissue extract. The next most abundant lipid class detected was that of nonpolar hydrocarbons, which eluted as a single peak after the void volume and represented  $12 \pm 0.7\%$  (SE;  $n = 5$ ) of total lipids.

## DISCUSSION

The evidence presented here indicates that the two major components of the vesicle envelope in free-living *Frankia* HFPCpI1 are bacteriohopanetetrol and a phenylacetate monoester of bacteriohopanetetrol ( $C_{43}H_{62}O_5$ ). No hopanoid esters have yet been reported in any organism, although several other classes of tetrol derivatives occur (16). A possible structure for the phenylacetate monoester is indi-

cated in Fig. 4, as a mono primary ester. We do not yet know definitively the position of the ester in the molecule. The same two major lipids were identified as the most abundant lipids in nodule-tissue extracts, demonstrating their occurrence and importance in symbiotic nitrogen fixation. Although the biological role of the amphiphilic hopanoid molecules as stabilizers of cell membranes is well characterized, the assembly of hopanoids into an extracellular layer represents an unusual adaptation.

The limitation of oxygen diffusion to the site of nitrogenase activity is a critical requirement for nitrogen fixation. In nitrogen-fixing symbioses, diffusion limitation may depend primarily on host tissue rather than microsymbiont adaptations, as in legume-*Rhizobium* root nodules (17, 18) or on both host and microsymbiont adaptations, as in the *Frankia* symbioses (5). Nitrogen-fixing prokaryotes in the free-living state have evolved numerous mechanisms to solve the oxygen problem, including elevated respiration, nitrogen fixation in microaerobic environments, temporal regulation (dark  $N_2$  fixation), compartmentation in specialized cells, or some interplay of more than one mechanism. In the case of *Frankia* and in certain cyanobacteria, delimitation of specialized nitrogen-fixing compartments by extracellular lipid layers appears to be a major element of oxygen protection. In *Anabaena* heterocysts, abundant envelope-specific glycolipids are synthesized during heterocyst differentiation (8, 19). A parallel situation appears to occur during vesicle differentiation in *Frankia*, although precise correlations between



hopanoid biosynthesis and vesicle development remain to be investigated. Nevertheless, although there are apparent structural and regulatory similarities between the vesicle envelope and the heterocyst envelope and there are clearly functional analogies, the two envelopes seem to have evolved from different biosynthetic pathways.

Our findings indicate that the phenylacetate monoester of bacteriohopanetetrol is vesicle specific or at least highly vesicle-enhanced, although bacteriohopanetetrol is present in both vesicles and hyphae in *Frankia*. The mechanism by which lamellae consisting of hopanoid molecules could limit oxygen diffusion may be one of structural exclusion of the oxygen molecule. The presence of the ester may enhance packing or modify charge relationships to facilitate oxygen exclusion. Some 16% of the purified envelope was not extractable as lipid. Although variability from experimental procedures probably accounts for this amount, we are investigating whether other classes of molecules are associated with the hopanoids in the envelope layers.

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